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GRANT NUMBER DAMD17-98-1-8016

TITLE: Preliminary Investigation of the Role of Cellular Immunity in Estrous Cycle Modulation of Post-Resection Breast Cancer Spread

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REPORT DATE: May 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20010419 090

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1999	Annual (20 Apr 98 - 19 Apr 99)

4. TITLE AND SUBTITLE
Preliminary Investigation of the Role of Cellular Immunity in Estrous Cycle Modulation of Post-Resection Breast Cancer Spread

5. FUNDING NUMBERS
DAMD17-98-1-8016

6. AUTHOR(S)
William J. Hrushesky, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albany Research Institute
Albany, New York 12208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

It is proposed to use this breast cancer of C_3H mice to determine whether or not hormone dependent immunocyte suppression is, in part, responsible for the mammalian fertility cycle modulation of the capacity of the cancer to spread after attempted surgical cure. These studies will determine which female sex steroid hormone(s) control(s) post resection metastatic spread. They will determine whether estrogen and/or progesterone affect depth and duration of surgery-induced natural killer cell activity suppression, numbers of NK and/or helper T and/or suppressor T cells following tumor resection. They will determine whether deleting specific immune cell types by the *in vivo* administration of antibodies directed against these cell types (asialo GM_1+CD_4+, CD_8+) abrogate the estrous cycle and sex hormone specific modulation of the post resection pattern of metastatic cancer spread. The results of the proposed studies, if negative, will exclude sex hormone modulation of cellular immune function as the most likely or important cause of the observed estrous cycle dependence of post surgical cancer spread. If these results demonstrate that NK and/or T helper and/or T suppressor cells are essential prerequisites of sex hormone modulation of post resection cancer spread, they will raise the hypothetical value of both neoadjuvant sex hormone and other specific cellular immune enhancement strategies prior to and/or immediately following primary breast cancer resection in order to potentially improve breast cancer cure frequency.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	22
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

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BODY:

During the first 12 months of this grant span, two approved tasks have been scheduled. These tasks relate to the first technical objective. Both of these tasks have been successfully completed. Task 3 was scheduled between 8 and 12 months. This task has been delayed. Task 4 originally scheduled for months 13-16 has, instead, been begun and partially completed.

Technical Objective #1 is as follows:

Technical Objective #1: Establish tumors, followed by surgical resection of tumors in mice at each of four estrous cycles and/or *in vivo* hormonal modulations, followed by determination of metastatic recurrence.

The entire sequence from tumor inoculation to the final endpoint of tumor recurrence is 5-6 weeks long. In order to be able to accurately perform tumor and surgical procedures and necropsies and to maintain the same age and circadian time for each experimental group and procedure, only groups of 20-30 mice can be handled at a time. Therefore, experimental groups will be staggered over time to achieve these goals.

Task 1: Months 1-6: Resect tumors in intact mice at one of four fertility cycle stages with no hormone modulation; follow up tumor recurrence with lung metastasis (20-30 mice at a time; total n=100 mice). Ongoing data analysis.

The completion of this task has entailed hiring and training a new post-doctoral associate and re-training a technician. Each of these individuals is now proficient in the tumor biology necessary to complete all aspects of the planned studies.

The biological studies scheduled under Task 1 have been completed and confirm earlier work demonstrating estrous cycle dependence of post resection metastatic potential.

In addition to the proposed tumor biology, tumors resected at specific estrous cycle stages have been frozen in liquid nitrogen for future quantitative RT-PCR of mRNAs which modulate cellular immunity.

Another addition to these studies in the preparation of the other half of each of these tumors for immunocytochemical assessment of macrophage, T cell subset and NK cell number and distribution in these estrous cycle specified tumor specimens.

Task 2: Months 6-8: Resect tumors in OVX mice with no hormone modulation (total n=25 mice). Data analysis.

All biological studies listed above have been all but completed in groups of OVX mice. This estrogen and progesterone-less state serves as an important control.

Task 3: Months 8-12: Ablate hormones (estrogen, progesterone) in intact mice with tumors using pellets with ICI 182780, RU486. Resect tumors. Follow up tumor recurrence with lung metastasis (n=25/group, 3 groups, total n=75 mice). Ongoing data analysis. Interim data analysis with preparation of data for presentation at cancer meetings.

Task 4: Months 13-16: Ablate hormones (estrogen, progesterone, prolactin) in OVX mice with tumors using pellets with ICI 182780, RU486. Resect tumors. Follow up tumor recurrence with lung metastasis (n=25/group, 3 groups, total n=75 mice). Ongoing data analysis.

Other hormone states have been studied to a limited extent. Now that trained personnel are in place, these studies will proceed more rapidly in year 2. Several of the goals listed under Task 4 have been completed early while Task 3 remains uncompleted as yet.

KEY RESEARCH ACCOMPLISHMENTS:

The most important accomplishment completed to date has been to get the commitment of an experienced junior investigator to take on this project in collaboration with Dr. Wood and myself. This individual was recruited from a world class reproductive biology background and brings unique skills, training and perspective to this project, synergizing reproductive and tumor biology. This collaborative chemistry has added to our capacity to understand the biology of the host-breast cancer balance.

This individual, Kathleen Bove, Ph.D., brings to the table expertise in quantitative RT-PCR, which now allows us to inspect the molecular mechanisms by which hormones coordinate immunologic function and the host-breast cancer balance at the mRNA level. This new capability adds an entirely new dimension to our work and will substantially alter our approach in years 2 and 3.

Cancer growth and spread is an intricate process dependent upon both the tumor and the host. Of particular interest to this laboratory is the role of the fertility cycle, and more specifically cyclic changes in steroid hormone levels, in tumor growth and metastases. Previous studies from our laboratory, using a primary, transplantable C_3H murine mammary carcinoma, have documented that breast cancer growth rate and post-resection metastatic behavior each change reproducibly during the estrous cycle. Prior work has also demonstrated that post resection cancer spread depends upon the time within the estrous cycle an advanced transplanted cancer is resected. 12-32% cure rates were seen in these studies. That early work described proestrus and estrus as optimal times for resection and metestrus and diestrus as particularly disadvantageous times for cancer surgery. Data presented here further examine the role of the estrous cycle in post-resection metastatic spread. This current work validates vaginal smear determined estrous cycle stage with uterine weight. A primary, transplantable, mammary carcinoma, was resected for surgical cure in young, cycling C_3HeB/FeJ female mice at each of 4 fertility cycle stages. A fifth group of oophorectomized (ovx) animals were also used. Following surgical resection, this carcinoma metastasizes to the lungs. In two, large, independent studies of earlier stage cancers a 96% surgical cure frequency occurs when the tumor is resected during estrus (peak but falling progesterone levels). The second best surgical cure rate is achieved when tumors are resected during metestrus (79% overall cure rate) while fewer tumors resected in diestrus or proestrus mice are cured (30-50%). We also show, for the first time, that there is no survival advantage conferred to ovx animals, suggesting a role for circulating E_2 and P_4 levels in the metastatic process.

Cancer growth and metastasis are dependent upon many complicated and inter-related processes. These may reside primarily with the tumor itself (e.g. hormone receptors, tumor suppressor and oncogene expression, production of autocrine growth factors and receptors, cell cycle/apoptosis dysregulation). Others arise primarily in the host (e.g. paracrine and endocrine factors, age, sex, fertility cycle stage, surgical stress and wounding, immune status). Different hormonal milieus associated with differences in sex, age, and fertility cycle stage, may alter tumor behavior through either direct effects upon tumor cells themselves and/or through effects upon host dependent processes that then less directly modulate tumor behavior. Prominent sex differences in the clinical cancer outcome following surgery and/or cytotoxic chemotherapy have been well described.[1] Several retrospective studies have shown that there is a distinct 10-year survival advantage for premenopausal women who undergo surgical resection of their breast tumors during the luteal phase compared to the follicular phase of the menstrual cycle (reviewed in Hagen et al., 1998).[2] We are particularly interested in the potential influence of reproductive hormones, such as estradiol (E_2) and progesterone (P_4), on the stress and wounding response induced by surgical resection, as reflected by the subsequent rate of post resection metastatic spread.

Tumor metastasis is ultimately responsible for most cancer deaths.[3] Successful treatment occurs more often in tumors which have not metastasized at the time of diagnosis than in those which have already spread when the tumor is detected.[4] A better understanding of the process of metastasis is needed in order to improve upon the survival of patients with cancer.

We investigated the influence of the estrous cycle on breast tumor surgical cure and metastatic potential, using a primary, transplantable, mammary carcinoma, resected for surgical cure from young, cycling C_3HeB/FeJ female mice at each of 4 fertility cycle stages to better define the precise estrous cycle stage for optimal surgical cure. Oophorectomized (ovx) animals were also used to determine the effect of minimal E_2 and P_4 levels on this phenomenon. This carcinoma, following resection, metastasizes to the lungs.

Our prior work in this model system was done at a stage in tumor growth when an average 25% of resected mice were apparently cured.[5] That work demonstrated superior outcomes for mice resected during proestrus (32% cure) and estrus (25% cure) while the worst outcomes were experienced in mice resected during metestrus (12% cure) and diestrus (22%

cure). Theses studies did not validate estrous cycling by measuring uterine weights. The current studies were initiated at earlier stages of tumor growth in order to see if the estrous cycle dependence of cure was relevant at more curable stages of cancer growth. In these studies we also measured uterine weights to validate the cycle stage of cancer resection.

Animal and Tumor Model: *Animals.* 2 separate experiments were performed. The first included 112 animals and the second included 100 sexually mature, female C_3HeB/FeJ mice (Jackson Laboratories), 8 wks of age, housed 4/cage, alongside singly housed male mice, to enhance estrous cycling as in our previous studies.[6,7] All animals were kept on lighting schedules with 12h light alternating with 12h of dark with food and water freely available. Time of day (circadian time) is referenced to hours after light onset (HALO). In the second study, bilateral oophorectomy was performed (n=20) at 10 wks of age. Animals were allowed to recover for 2 weeks prior to tumor injection. Confirmation of oophorectomy was accomplished through serial vaginal diestrus cytology. *Tumor.* The breast cancer primary tumor (B. Fisher, Univ Pittsburgh) originated spontaneously in a female C_3H mouse and was passed *in vivo* in C_3HeB/FeJ female mice. Tumors were harvested under sterile conditions and tumor cell suspensions were made by gentle grinding of minced tumor pieces over a stainless steel mesh into Medium 199 (Gibco-BRL). Tumor cells were inoculated subcutaneously at $2x10^4$ viable cells in the right hind leg during the early activity phase (14 HALO) in both studies. Tumors were measured daily (length, width, height) by the same individual, using calipers. Tumors were excised from animals in one of four estrous stages and from oophorectomized animals (n=15/stage), at an average size of $900mm^3$ by surgical removal of the right leg. In the first study, surgical tumor resection occurred at two different times of day (during the early activity phase (14 HALO) and in the early sleep phase (2 HALO)). Time of day of resection was found not to affect tumor metastatic potential, so in the second study tumors were resected only at 14 HALO. Animals were monitored daily for local tumor recurrence. Animals with local tumor recurrence at the site of resection (57% study 1, 48% study 2) were considered surgical failures and were not included in further analyses. All animals were sacrificed (when 5% of the animals died from lung metastases) and autopsied for the presence or absence of lung metastatic lesions. Uteri were removed, weighed and stored at -80°C. Serum was recovered and stored at -80°C.

Fertility Phase Determination: Daily vaginal smears were done using sterile saline washings, stained with Diff Quik (Baker) and were read by one individual using standard criterion.[8] Slides from each mouse were read in a daily sequence to determine the progression of cycling and classify smears as proestrus (P), estrus (E), metestrus (M), and diestrus (D). Estrous cycling was

determined daily, starting 4 days prior to tumor inoculation until tumor resection to ensure regular cycling in each mouse and the assignment of estrous stage at the time of tumor resection. Uterine wet weights were recorded in a separate group of tumor bearing animals for validation of estrous cycle stage classification.

Reverse Transcription-Polymerase Chain Reaction: Uteri were rapidly collected, homogenized and total RNA recovered (TRIZOL,Gibco-BRL). First strand cDNA was generated from 1.0 μg of total RNA using SuperScript II reverse transcriptase (Gibco-BRL). Quantitative PCR was performed according to the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer) using ^{32}P-labeled dCTP. Oligonucleotide paired primers for mouse ribosomal protein S16 and histone 3.2 were purchased from Gibco-BRL (Gaithersburg, MD). PCR samples were fractionated by electrophoresis on an 8% PAGE and quantitated by phosphorimage analysis (STORM 860, Molecular Dynamics). The linear range of amplification was determined for each primer pair. Results are expressed as the ratio of the gene of interest to control gene for each sample (ie., histone 3.2/S16).

Statistical Analyses: Parametric analyses: Numerical values for uterine weight were contrasted across the four estrous cycle phases, and also in the ovx state (second study) using one way ANOVA with SuperANOVA statistical software. Nonparametric analysis (Chi^2) analyses was used to determine whether the proportion of animals cured across the cycle was randomly distributed using SPSS statistical software.

Local Tumor Recurrence

Overall local recurrence occurred in 53% of the mice. In study 1, the proportion of local recurrences was not affected by the timing of resection within the estrous cycle (Figure 1A; $X^2 = 2.43$, $p = 0.488$). This observation was also found in study 2 (Figure 1B; $X^2 = 6.7$, $p = 0.153$). The combined data from these two studies also supports this finding (Figure 1C; $X^2 = 7.25$, $p = 0.123$).

Metastatic Spread of Tumors

In the first study, the greatest surgical cure (100%) was found in animals whose tumors were resected during the estrus phase of the fertility cycle (Figure 2A). Those animals with tumors resected during metestrus faired second best with 88.5% cure rates. Animals resected in either diestrus or proestrus had the lowest surgical cure rate (40% and 20%, respectively). Similar results were obtained when animals were resected at either 2 HALO or 14 HALO (Table 1)

Comparable results were obtained in the second study. There was a 93% surgical cure rate in those animals that had their tumors resected during the estrus phase of the cycle (Figure 2B). The second best surgical cure was found in animals whose tumors were resected during metestrus (67%). Surgical cure in animals who were resected during proestrus, diestrus or in ovx animals were similar (50%, 47%, 50% respectively).

The combined data from these two studies is shown in Figure 2C. Together these studies demonstrate that the fertility cycle stage at the time of tumor resection has a significant effect on surgical cure/metastatic spread (Chi^2: $F = 24.9$, $p<0.0001$).

To confirm our estrous stage classification we have also analyzed uterine wet weights and mRNA levels of a marker of uterine proliferation (histone H3.2) as a function of the estrous cycle (Figure 3). We found that uterine weight varies as a function of the estrous cycle: showing an estrogen-induced increase in uterine weight as demonstrated in the literature (Figure 3A). Also, mRNA levels for histone 3.2 vary as a function of the estrous cycle, paralleling known changes in uterine proliferation (Figure 3B).

It is logical to speculate that the microenvironment at and near the time of estrus (characterized by high but declining P_4 levels) is responsible for alterations in the gene expression patterns of factors controlling metastatic spread of this tumor. In the rodent, proestrus, the stage immediately preceding estrus, has a duration of 7 h and is characterized by large hormone fluxes as the ovary prepares for ovulation. The estrus phase of the cycle lasts 9-15 h.[9] Levels of the ovarian steroid E_2 rise early in proestrus to reach peak values, plateau by mid-proestrus and reach near basal levels by estrus; while P_4 reaches peak levels in late proestrus/early estrus, reaching basal levels by mid-estrus.[10,11,12] In addition, there are dramatic changes in pituitary hormone levels occurring at this time. Circulating levels of luteinizing hormone (LH) rise rapidly and peak in late proestrus and immediately decline to basal levels by estrus.[11] Follicle stimulating hormone levels peak mid-proestrus (simultaneously with LH), begin to decline and then show a second peak in early estrus.[12] Prolactin levels parallel LH levels, peaking in late proestrus and then rapidly declining to basal levels by estrus.[12] Such dramatic changes in the hormonal milieu from late proestrus to mid-estrus are likely to have an impact on tumor physiology. We have begun to analyze gene expression patterns in these tumors to determine if there is a 'metastatic' marker present in these tumors either turned on or turned off by changes in steroid hormone levels during certain stages of the fertility cycle. The fertility cycle stage dependence of post-resection breast cancer metastatic potential may arise, at least in part, from the sex hormone control of angiogenesis in dormant micrometastases.

These data differ from those previously reported in this laboratory. In earlier studies far fewer cures were achieved and the where the greatest surgical cure frequency was found in animals resected near or at estrus.[6] Ratajczak et al. classified animals into only 2 categories ("near estrus" and "post estrus") while we have performed a more thorough, classic, 4 stage estrous reading.[8] Also, we have confirmed the identification of the smears by examining corresponding uterine wet weight and a marker of uterine cellular proliferation (Histone H3.2). Uterine wet weights are consistently higher in proestrus and estrus as a result of the estrogen-induced increases in hyperemia and water imbibition (reviewed in Clark and Markaverich, 1988).[13] Estrogen has also been shown to increase proliferation in the uterus during these same time points.[13] The average cure frequency following resection was more than doubled in these studies indicating that the cancers resected in prior studies were more advanced. Other work

indicates that the host-cancer balance changes with tumor growth. It is possible that the hormonal milieu more favorable for less advanced cancers may differ from that for more advanced cancers.

Ongoing work attempts to determine which molecular pathways within the host tissue (soil) and tumor cells (seed), independently demonstrated to affect metastatic potential, are coordinated by estrous cycle phase and are reproducibly associated with either cure or metastatic cancer spread. Using tumor samples obtained from premenopausal women with breast cancer, along with menstrual data coupled with hormonal monitoring, Saad et al., examined mRNA levels of various genes whose expression has been associated with malignancy and metastatic potential.[14] Expression levels for two genes associated with tissue degradation and metastatic spread, cathepsin L and MMP-9 as well as p53 were found to be higher in tumors which had been resected during the follicular and periovulatory phases of the menstrual cycle compared to other phases of the cycle. These data showed that the malignant properties of breast tumors could cycle over the menstrual cycle.[14]

Gene products, which are candidates for mediation of the fertility cycle dependence of metastatic potential, emerging from these studies will ultimately be knocked out and/or over expressed. The effects of these genetic manipulations upon the host/cancer balance and its' estrous cycle control will yield cause and effect information that may one day be employed therapeutically.

Table 1

A: Time of Day Effect on Fertility Cycle-Regulated Surgical Cure

Time of Surgery:	*2 HALO*	*14 HALO*
	% Surgical Cure	
Estrous Stage at Resection:		
Proestrus	33% (1/3)	14% (1/7)
Estrus	**100% (5/5)**	**100% (7/7)**
Metestrus	86% (6/7)	100% (1/1)
Diestrus	0% (0/2)	50% (4/8)
	Chi^2, p	
	9.6, $p<0.022$	11.4, $p<0.01$

B: Time of Day Effect on Overall Surgical Cure

HALO at Resection	**% Surgical Cure**
2 HALO	71% (12/17)
14 HALO	50% (13/26)
	Chi^2, p
	0.83, $p<0.364$

FIGURE LEGENDS

Figure 1. The effect of estrous cycle stage at time of resection on local tumor recurrence. Percentage of animals, from each estrous cycle stage, with locally recurring tumors. **A**: Study 1. **B**: Study 2. **C**: Combined data from study 1 + study 2.

Figure 2. The effect of estrous cycle stage at tumor resection on surgical cure. Tumors were resected for cure from animals in each of the estrous stages. % Surgical cure in these animals is determined by the absence of metastatic lesions in their lungs. **A**: Study 1. **B**: Study 2. **C**: Combined data from study 1 + study 2.

Figure 3. Confirmation of estrous cycle stage classification. 3A: Uterine wet weights as a function of estrous cycle stage. 3B: RT-PCR of uterine histone 3.2 mRNA levels as a function of estrous cycle stage. PI units = phosphorimage units

Figure 1. The Effect of Estrous Cycle Stage at time of Resection on Local Tumor Recurrence

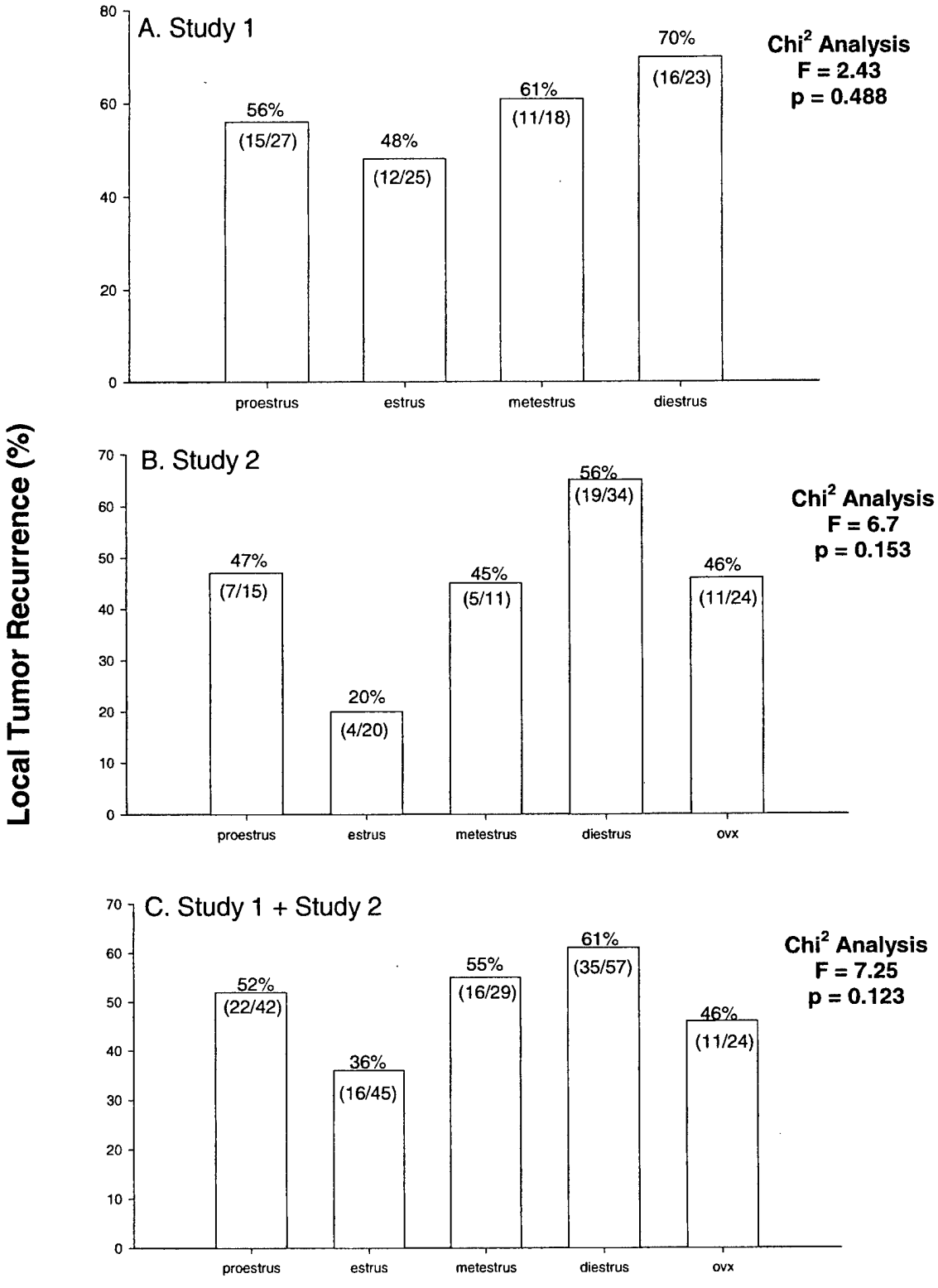


Figure 2. The Effect of Estrous Cycle Stage at Tumor Resection on Surgical Cure

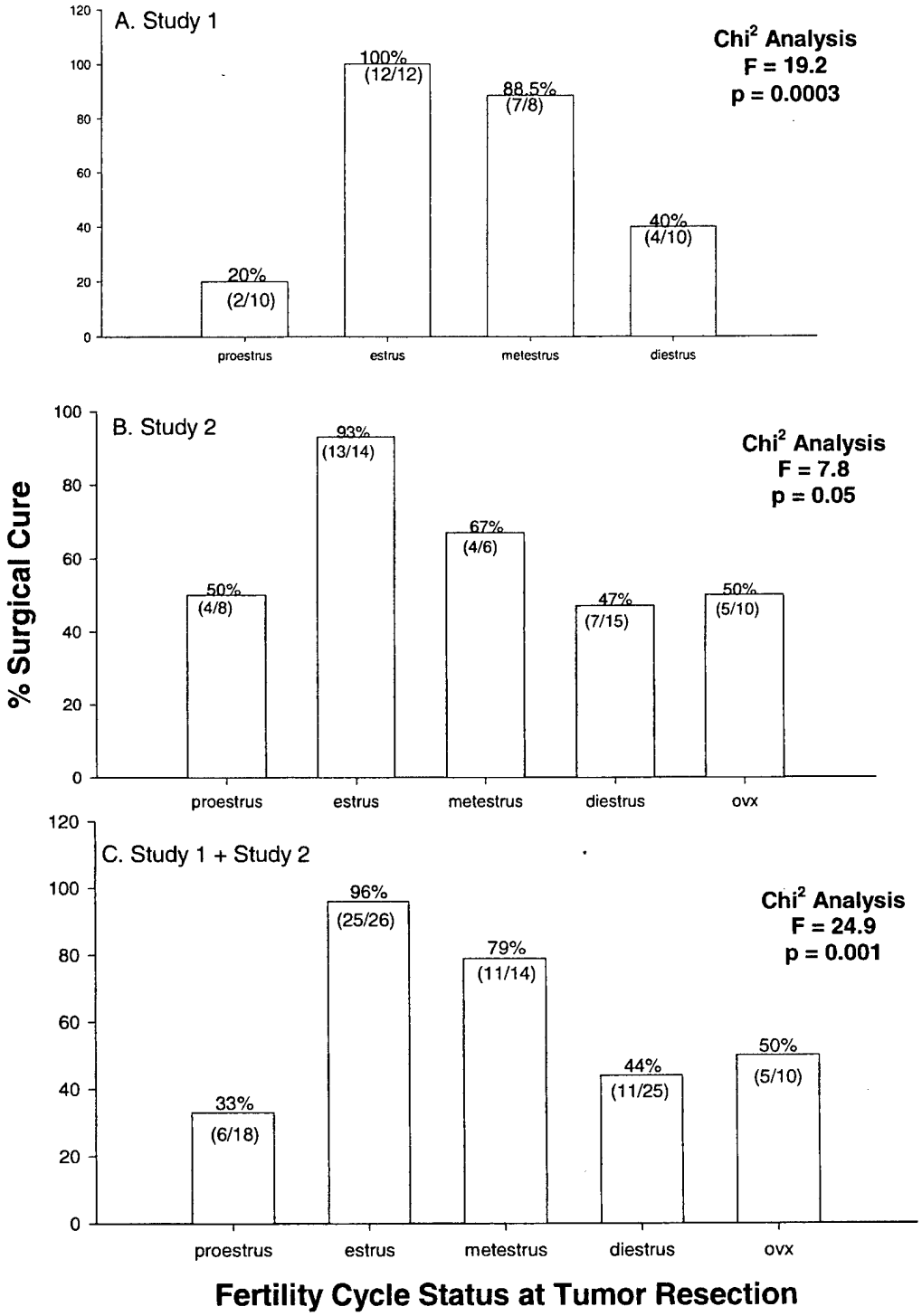
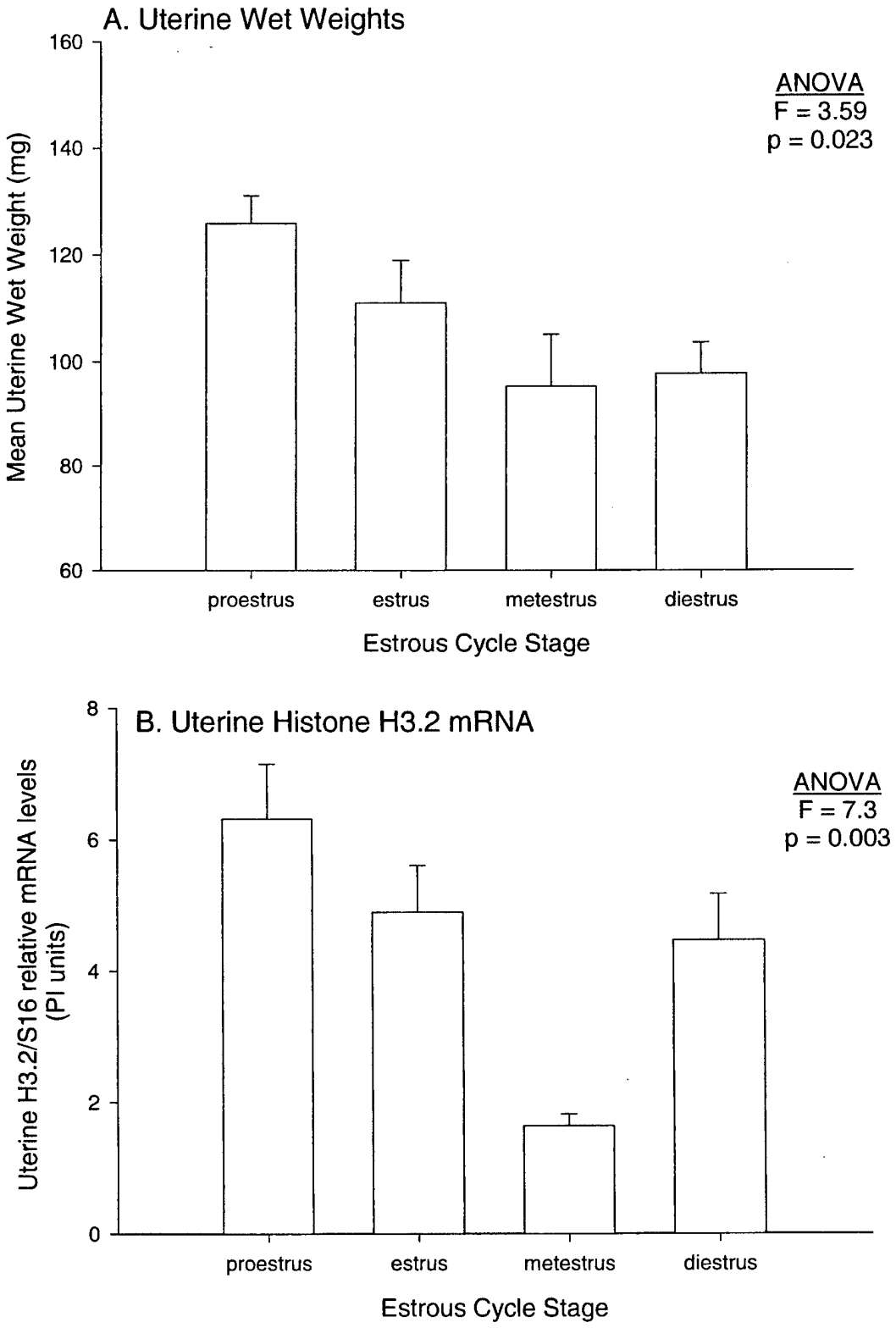


Figure 3. Estrous Cycle influence on Uterine Wet Weight and Histone H3.2 mRNA Expression



REPORTABLE OUTCOMES:

PAPERS

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CONCLUSIONS:

It is my conclusion that the team now in place for this project is well underway in understanding how immune function may mediate the estrous cycle stage (sex hormone) control of post resection metastatic potential. A genuine understanding of these connections will allow us to plan breast cancer resection at times within a young woman's cycle to maximize her prospects for cure and to devise hormono-immunological strategies to enhance the curability of all cancers resected for cure.

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